Preparative Methods of Isolating Bitter Peptides from Cheddar Cheese†

Kai-Ping D. Lee and Joseph J. Warthesen*

Minnesota−South Dakota Dairy Foods Research Center, Department of Food Science and Nutrition, University of Minnesota, St. Paul, Minnesota 55108

A preparative procedure including water extraction, membrane ultrafiltration, and reversed-phase HPLC separation to isolate bitter peptides in Cheddar cheese was developed. The most bitter fractions in cheese samples were in a molecular weight range of either between 500 and 3000 or >3000. When equal amounts of the lyophilized bitter fraction were incorporated back into cheese, reduced-fat Cheddar cheese samples had a more pronounced increase in bitterness intensity than full-fat cheese samples. Sensory evaluation on the eluants of preparative HPLC revealed that bitterness was found in various places along the separation, although eluants in the early region of chromatograms were generally more bitter. Most of the isolated bitter peptides contained a high level of hydrophilic amino acid residues such as glutamic acid/glutamine and serine. Hydrophobic amino acid residues, such as leucine, isoleucine, and proline, were high in some bitter peptide fractions, but phenylalanine and tyrosine were absent in all isolated bitter peptides.

Keywords: *Preparative methods; bitter peptides; Cheddar cheese*

INTRODUCTION

Bitter flavor is a common defect in Cheddar cheese. Some bitterness is probably a normal constituent of cheese flavor and may be undetected or even desirable. However, excessive bitterness is objectionable and of considerable economic significance. It is acknowledged that the bitter flavor produced in Cheddar cheese is the result of some oligopeptides with certain properties arising from the degradation of casein (Lowrie and Lawrence, 1972). Mixtures of various organic solvents have often been used by researchers to extract bitter peptides of interest from the cheese matrix (Champion and Stanley, 1982; Hamilton et al., 1974; Harwalkar and Elliott, 1971). Other researchers (Edwards and Kosikowski, 1983; Richardson and Creamer, 1973) have used water extraction to prepare a water-soluble nitrogen fraction containing bitter peptides from Cheddar cheese. For subsequent sensory evaluation, water is a preferred solvent because of the safety concerns for human subjects involved in bitter peptide tasting. In the study of Kuchroo and Fox (1982), numerous parameters affecting the extraction of water-soluble nitrogen were investigated. Aston and Creamer (1986) demonstrated that the water-soluble nitrogen is an important contributor to nonvolatile flavors in cheese.

A procedure of fractionating water-soluble nitrogen to identify key compounds generated from proteolysis in Irish Cheddar cheese was reported by Singh et al. (1994). Membrane ultrafiltration following crude extraction has often been used because of its capacity to separate molecular weights in the range of interest. Liquid chromatography, such as gel filtration or ion-exchange chromatography, and gel electrophoresis, also have been employed to further separate compounds in the filtrates (Champion and Stanley, 1982; Singh et al., 1994). However, these techniques do not have sufficient resolving power to provide the desirable separations. In a previous work (Lee and Warthesen, 1996), a food grade mobile phase system for HPLC was developed to provide the necessary resolution of peptide separations. To extend the scale of analytical analysis, preparative HPLC could be incorporated so a sufficient amount of peptides can be obtained for subsequent sensory evaluation and composition characterization. In recent years, capillary electrophoresis has become a promising analytical tool in many applications of biomolecules and pharmaceuticals, especially in the separation of proteins and peptides (Grossman et al., 1989; McCormick, 1988). With the techniques of both preparative and analytical HPLC combined with capillary electrophoresis, isolation of high-purity bitter peptides in large quantities prior to the final characterization could be achieved.

Although some peptides from Cheddar cheese have been characterized as bitter (Hamilton et al., 1974; Richardson and Creamer, 1973), their bitterness perception was usually examined in water solution. Attempts to reincorporate the bitter peptide fractions into cheese samples to ensure whether they actually cause bitterness in cheese have not been reported. The objectives of this study were to establish a quantitative method coupled with analytical techniques to isolate bitter peptides from Cheddar cheese, to confirm their sensory effect in the cheese matrix, and then to characterize the composition of the bitter peptides.

MATERIALS AND METHODS

Cheddar Cheese Samples. For bitter peptide extraction studies, five full-fat and one reduced-fat Cheddar cheese samples (cheeses 1−6) with ages from 10 to 27 months were obtained from commercial sources and the pilot plant in the Department of Food Science and Nutrition, University of Minnesota. The cheeses were determined to be bitter in preliminary sensory tests. The protein, fat, pH, salt, and moisture compositions of each cheese sample were measured in triplicate. The Kjeldahl and Mojonnier methods following the AOAC procedures (1984) were used to determine protein and fat contents. A Corning flat surface combination electrode (Corning Science Products, Medfield, MA) was used to measure

† Published as Paper 21 958 of the contribution series of the Minnesota Agricultural Experimental Station.

* To whom correspondence should be addressed [fax (612) 625-5272; e-mail jwarthesen@che2.che.umn.edu].

0021-8561/96/1444-1058$12.00/0

pH on the well-mixed ground cheese sample. The salt and moisture contents also were determined using the AOAC methods (1984).

Four other Cheddar cheese samples (cheeses 7−10), two full-fat and two reduced-fat with ages of 4, 16, 2, and 9 months, were obtained from the pilot plant in the Department of Food Science and Nutrition at the University of Minnesota. These cheese samples were used in a confirmation study to determine the sensory effect after the addition of a bitter peptide fraction into cheese matrix. Each cheese was stored at 4 °C and analyzed within 2 weeks after it was obtained.

Preparation of Molecular Weight Fractions. A procedure modified from the method of Kuchroo and Fox (1982) was used to extract the water-soluble nitrogen fraction from Cheddar cheese samples that were determined to be bitter by the sensory panel. One hundred grams of cheese was grated and blended at room temperature with 200 mL of deionized distilled water using a Sorvall Omni Model 17105 mixer (DuPont Instruments, Newton, CT) for 2 min on the speed setting of 3. The slurry was centrifuged for 30 min at 18000*g*, 4 °C. The fat layer on the top was discarded, and the supernatant of water extract was retained. The precipitate was reextracted with water following the same procedure. Supernatants were combined, filtered through glass wool, and ultrafiltrated using an Amicon Model 8400 ultrafiltration unit (Amicon Inc., Beverly, MA) until about 10% (v/v) of the extract remained. A volume of water equal to the retentate was added to the extract and filtration continued until about 10 mL of retentate remained. Molecular weight cutoff membranes of YC05 and YM3, purchased from Amicon, were used to separate the extract based on the molecular weight cutoffs of 500 and 3000, respectively. The obtained three molecular weight fractions were frozen, freeze-dried, and stored at −20 °C for further use.

Bitterness Confirmation in Cheese Matrix. Twenty milligrams of a lyophilized bitter fraction in the molecular weight range of 500−3000 was mixed with 1 mL of water and stirred with 1 g of shredded Cheddar cheese at room temperature. Sensory evaluation of bitterness was immediately carried out on each cheese sample with and without the incorporation of the bitter molecular weight fraction.

HPLC Analyses. The peptide separation using HPLC was conducted in two parts. In the preparative chromatography, the HPLC was equipped with a Vydac C18 Model 201TP1022 preparative column (10-μm particle size, 25 cm × 2.2 cm i.d.) (Vydac, Hesperia, CA). The lyophilized molecular weight fractions dissolved in distilled water (100 mg/mL) were filtered through 0.45-μm filters and injected into the HPLC using a Rheodyne Model 7125 injector (Rheodyne Inc., Cotati, CA) with a 1-mL injection loop. Solvent A contained 0.1% (v/v) HCl (Food Chemical Codex grade, Spectrum Chemical Mfg. Corp., Gardena, CA) in HPLC grade water, and solvent B contained 0.1% (v/v) HCl and 90% (v/v) food grade absolute ethanol (University Storehouse, St. Paul, MN) in HPLC grade water. Peptides were eluted with a gradient of 0−50% solvent B over 50 min and 50−80% solvent B in 10 min at a flow rate of 5.0 mL/min, using a Spectra-Physics Model P2000 pump (Spectra-Physics Analytical Inc., Fremont, CA). The percentage of solvent B in the mobile phase was then decreased to 0% over another 10 min at the same flow rate. Separation profiles were monitored by UV absorption at 220 nm with a Spectra-Physics Model UV150 detector. Peak area was measured using a Hewlett-Packard Model 3380A integrator (Hewlett-Packard Co., Naperville, IL).

Starting 10 min after a sample injection, the void time of the preparative column, eluants were collected every 5 min over the 60-min run using a Gilson Model 210 fraction collector (Gilson Medical Electronics, Inc., Middleton, WI). The collection was completed with five repeated HPLC injections and 12 fractions each with a final volume of 125 mL. The liquid in the eluants (ethanol and water) was lyophilized, and each fraction was reconstituted with distilled water to 10 mL for sensory evaluation.

In the analytical chromatography, a Vydac C18 Model 201TP54 analytical column (5-μm particle size, 25 cm × 4.6 mm i.d.) was used to separate peptides in selected eluant fractions collected from the preparative chromatography. Chromatography conditions were the same as described in the preparative separation except a variable gradient elution was performed depending on the injected eluant fraction. The flow rate was 1.0 mL/min, and the sample injection volume was 100 μL. Eluants of selected separated peaks were collected for 20 repeated HPLC injections, lyophilized, reconstituted with water to 10 mL, and tasted by the sensory panel for bitterness.

Sensory Evaluation. Twenty subjects from the Department of Food Science and Nutrition at the University of Minnesota participated in a screening session to determine their sensitivity toward bitterness and reproducibility on the bitterness rating. The thresholds of quinine sulfate and 6-*n*-propylthiouracil, purchased from Sigma Chemical Co. (St. Louis, MO), were measured for each subject according to the methods of Glanville and Kaplan (1965) and Anliker et al. (1991). Five individuals were selected to form a sensory panel. Their average thresholds of quinine sulfate and 6-*n*-propylthiouracil were 7.32×10^{-4} and 2.23×10^{-5} mM, respectively. A rating sheet with unstructured 160-mm lines was provided to rate the sample bitterness, and 0 at the left represented nonbitter. The degrees of bitterness intensity were divided into slightly, distinctly, very, and extremely bitter, which were evenly distributed on the line, and each degree represented a quinine sulfate concentration of 2.9×10^{-3}, 5.8×10^{-3}, 1.2×10^{-2}, and 2.4×10^{-2} mM, respectively. Water and a quinine sulfate solution of 2.9×10^{-3} mM were provided as standards at each tasting session. Panelists were requested to rinse their mouths thoroughly with water before tasting and to keep the sample in the mouth for about 10 s before rating. A 1.5 cm^3 cube (about 3 g) of the six Cheddar cheese samples and a volume of 1 mL of molecular weight fraction solutions (20 mg/mL of distilled water) were used in bitterness evaluation for each subject sensory tasting. In the bitterness confirmation study, 1 g of shredded cheese was provided to each panelist at each tasting; 1 mL of reconstituted eluants was used to measure bitterness intensity of the collected HPLC peaks. All of the sensory tasting was conducted in duplicate. Sensory data were analyzed by analysis of variance with the pair and Tukey's multiple comparison tests to separate means using the SAS (1985).

Purity Identification with Capillary Electrophoresis. Purity confirmation of peak eluants collected from analytical HPLC was performed using a Spectra PHORESIS 500-capillary electrophoresis apparatus (Spectra-Physics Analytical Inc., San Jose, CA) controlled by a PC computer that operated Spectra-Physics capillary electrophoresis software. The capillary cassette was threaded with an uncoated 75-μm i.d. fused-silica 70-cm column. Samples filtered through 0.2-μm filters were introduced into the capillary with a hydrodynamic injection mode for 3 s. Electrophoretic runs were conducted with an applied voltage of 15 kV. On-line detection at 200 nm was used over the 20 min run, and external temperature of the capillary column was maintained at 30 °C. Sodium phosphate buffer (50 mM, pH 7.0) was used to carry samples through the capillary column. Each run was followed by 3-min individual rinses of 0.1 N NaOH, distilled water, and sodium phosphate buffer (50 mM, pH 7.0) for the capillary conditioning.

Amino Acid Composition of the Isolated Peptides. Purified bitter peptides isolated from the eluants of analytical HPLC and confirmed by capillary electrophoresis were hydrolyzed in 6 N HCl for 24 h at 110 °C before the composition analysis using a Beckman Model 6300 amino acid analyzer (Beckman Instruments Inc., Palo Alto, CA). Data were acquired and analyzed on a Beckman Gold Data System instrument.

RESULTS AND DISCUSSION

Cheese Composition and Bitterness Intensity. The bitterness intensity and compositional analysis of six Cheddar cheese samples (sample 4 was manufac-

Table 1. Bitterness Intensity and Composition Analysis of Six Cheddar Cheese Samples

	cheese sample					
	1	2	3	4	5	6
bitterness intensity (mm[d])	129.80[a]	94.55[b]	92.40[b]	88.95[b,c]	63.50[b,c]	57.60[c]
protein (%)	23.69[b]	24.37[b]	25.81[b]	31.90[a]	25.39[b]	26.17[b]
fat (%)	36.39[a]	34.24[a]	34.33[a]	15.90[b]	35.97[a]	33.30[a]
moisture (%)	34.87[b]	34.39[b]	32.89[b]	46.08[a]	32.50[b]	33.89[b]
salt (%)	1.30[b]	1.46[a,b]	1.74[a]	1.24[b]	1.34[b]	1.36[b]
pH	5.37[a]	5.37[a]	5.19[b,c]	5.08[c]	5.08[c]	5.26[a,b]
salt-in-moisture ratio (%)	3.70[b]	4.20[b]	5.30[a]	2.20[c]	4.10[b]	4.00[b]

[a−c] Means in the same row with like superscripts do not differ ($P > 0.05$). [d] mm on 160-mm scale; 0 = nonbitter.

Table 2. Bitterness Intensity (mm) of Three Molecular Weight Fractions from Cheese Samples with High Bitterness Intensity

	molecular weight		
cheese sample	<500	between 500 and 3000	>3000
1	41.11[c]	69.94[a]	54.83[b]
2	48.00[b]	57.10[a,b]	66.47[a]
3	53.23[a]	62.33[a]	58.16[a]

[a−c] Means in the same row with like superscripts do not differ ($P > 0.05$).

tured as a reduced-fat product) are presented in Table 1. Cheese 1, which exhibited the most intense bitterness perception, had the highest amount of fat and the lowest protein; however, these composition differences were not significant ($P > 0.05$) when compared to those of other full-fat cheese samples. Except for cheese 4, high moisture and low salt contents of cheese 1 resulted in the lowest salt-in-moisture ratio compared to other full-fat cheese samples. Thomas and Pearce (1981) noted that the salt-in-moisture range of 4−6% was an important characteristic of high-quality cheese. According to Visser et al. (1983), salt was an important factor influencing the formation and degradation of bitterness in cheese because it governed the extent of proteolysis. It has been reported that reduced-fat cheese had a prevalent problem in bitterness (Deane and Dolan, 1973; Olson, 1984) because of a low salt-in-moisture content (affecting proteolytic enzyme activity) or a minimized bitterness masking effect. However, the reduced-fat cheese product was not as bitter as cheese samples 1−3. The highest pH was found in the two most bitter cheese samples (cheeses 1 and 2), but insufficient data limited establishment of a relationship between pH and bitterness intensity.

From the results in Table 1, the relation of composition and bitterness of cheese was not found, nor was there a trend of bitterness intensity to the rank order of any compositional component. The bitterness of cheese needs to be explained by factors other than the proximate composition and pH. Effects of the proteolytic enzyme activity from starter cultures or secondary microflora and the accumulated bitter peptides with a concentration exceeding the detection threshold might be more directly related to bitterness intensity of cheese.

Molecular Weight Range and Bitterness Intensity. Cheddar cheese samples 1−3 with high bitterness scores were selected for extraction and subsequent fractionation of peptides based on molecular weight. Bitterness intensities of three molecular weight fractions for each cheese sample are shown in Table 2. Based on sensory analyses, the fraction of peptides from cheese 1 with a molecular weight between 500 and 3000 was the most bitter fraction which was significantly different in bitterness ($P < 0.05$) from the other two fractions. For cheese 2, the most bitter fraction was in the molecular weight range >3000, although significant differences in bitterness were not observed ($P > 0.05$) with the fraction of a molecular weight between 500 and 3000. No significant differences ($P > 0.05$) in bitterness were found between any molecular weight fraction in cheese 3 even though the fraction with a molecular weight between 500 and 3000 had the highest bitterness score. It was noted that the fraction of peptides with a molecular weight <500 received the lowest bitterness scores in all three cheese samples. It should also be noted that the molecular weight fractions are based on manufacturer specifications and can vary in range of molecular weight cutoff and degree of filtration efficiency.

Sullivan and Jago (1972) concluded that most bitter peptides were in the range from 2 to 23 amino acids, even though a larger bitter peptide consisting of 27 residues was later reported by Clegg et al. (1974). These reports suggested that a fraction of peptides with a molecular weight from 500 to 3000 (about 4−27 amino acid residues) might have a potential to be a bitter fraction extracted from a bitter cheese. This postulation was supported by current results that the fraction containing peptides with a molecular weight from 500 to 3000 exhibited the strongest bitterness intensity in cheese 1 and also a relatively strong response in the other two cheese samples. Bitter peptides with a molecular weight >3000 have not been reported to date. Results showing the fraction of peptides with a molecular weight >3000 revealed the strongest bitterness in cheese 2 could be attributed to an incomplete ultrafiltration so residual bitter peptides smaller than molecular weight of 3000 might have remained in the retentate. Membranes with an imprecise molecular weight cutoff also might result in some smaller bitter peptides retained in a higher molecular weight retentate. According to Lowrie and Lawrence (1972), bitterness of cheese could decrease when bitter peptides were degraded to smaller peptides and amino acids. Adler-Nissen (1984) further demonstrated that peptides are usually more bitter than the corresponding mixture of free amino acids. These observations might support the current result that the lowest bitterness intensity was found in the fraction with a molecular weight <500. Fractionation by molecular weight was used in later studies because the HPLC separation was too complex for good resolution without a preliminary separation by membrane.

Effect of the Bitter Peptide Fraction in Cheese. Table 3 contains the bitterness scores of cheddar cheese samples 7−10 with and without the addition of the bitter peptide fraction (molecular weight range of 500−3000) extracted from cheese 1. Significant increases ($P < 0.05$) in bitterness intensity of two reduced-fat cheese samples (cheeses 9 and 10) were observed after the addition of bitter fraction. A slight decrease of intensity

Table 3. Bitterness Scores (mm) of Cheddar Cheese Samples with and without the Addition of a Bitter Molecular Weight Fraction with a Molecular Weight between 500 and 3000 from Cheese 1

	cheese sample			
	7	8	9	10
bitterness intensity of cheese samples				
with addition	78.38[a]	17.25[a]	61.63[a]	36.75[a]
without addition	84.75[a]	22.13[a]	42.63[b]	18.87[b]

[a,b] Means in the same column with like superscripts do not differ ($P > 0.05$).

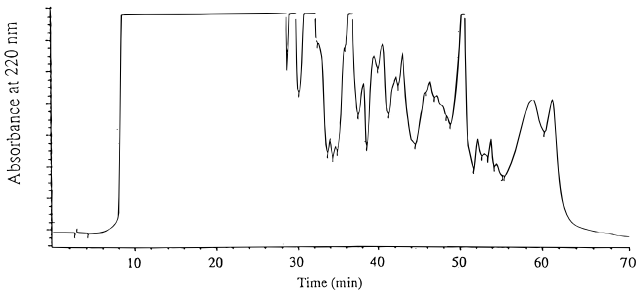

Figure 1. Separation profile of the fraction of peptides with a molecular weight between 500 and 3000 from cheese 1 using preparative HPLC.

in bitterness of full-fat cheese samples (cheeses 7 and 8) was found, but no significant differences were observed ($P > 0.05$) between the bitterness scores of full-fat cheese samples before and after incorporation of the molecular weight fraction. Because the amount of bitter fraction added to cheese was small and composed of a number of peptides, not just bitter peptides, the lack of an increase in bitterness of full-fat cheese samples after mixing with the bitter fraction might be attributed to the masking effect of fat along with an insufficient concentration of the bitter peptide fraction added. Results in Table 3 demonstrate that the fraction containing bitter peptides had a more pronounced effect on the bitterness intensity in reduced-fat than full-fat cheese. Because sensory evaluation on cheese bitterness was conducted immediately after the incorporation, degradation of bitter peptides in the molecular fraction by proteolytic enzymes in cheese was not likely to have occurred.

Preparative HPLC Analysis and Eluant Tasting. The separation profile of the fraction of peptides with molecular weight between 500 and 3000 from cheese 1 using preparative HPLC is demonstrated in Figure 1. Coelution of peaks was observed along the separation because of a large injection volume and a concentrated sample. The relationship between the sensory data on bitterness intensity of every 5-min eluant fraction and the elution order for two molecular weight fractions is shown in Table 4. No particular pattern of bitterness intensity to the elution order of eluants was noted. On the contrary, the eluants determined to be bitter were found in various places along the separation, indicating that chemical characteristics of the separated bitter compounds might be quite different from each other. Although some late-eluting fractions such as fraction 9 or 12 were determined to be relatively bitter, depending on the cheese and molecular weight fraction, eluant fraction 1 was generally the most bitter in sensory tasting. Because the bitter compounds eluted both early and later in the chromatogram, the bitter peptides fractionated in this study might possess both hydrophilic and hydrophobic properties.

Table 4. Bitterness Intensity (mm) of Every 5-min Eluant Fraction for Both Fractions with Molecular Weight between 500 and 3000 as well as >3000 from Cheeses 1 and 2

	molecular weight range			
	cheese sample 1		cheese sample 2	
eluant fraction	between 500 and 3000	>3000	between 500 and 300	>3000
1	60.25[a]	56.15[a]	56.47[a]	63.93[a]
2	23.15[b]	22.10[b,c]	47.13[a,b]	27.37[d]
3	26.45[b]	13.55[c]	22.23[c]	29.97[d]
4	35.40[b]	21.70[b,c]	27.83[c]	46.03[a,b,c]
5	13.75[b]	12.80[c]	38.40[a,b]	29.93[d]
6	22.40[b]	20.12[b,c]	26.93[c]	37.07[c,d]
7	30.00[b]	21.35[b,c]	18.27[c]	27.50[d]
8	20.40[b]	32.00[b]	16.77[c]	41.63[b,c,d]
9	21.90[b]	56.80[a]	27.93[c]	50.47[a,b]
10	22.00[b]	26.00[b,c]	32.83[b,c]	35.30[c,d]
11	23.90[b]	24.10[b,c]	22.70[c]	22.73[d]
12	20.85[b]	42.00[a,b]	29.67[b,c]	55.77[a,b]

[a–d] Means in the same column with like superscripts do not differ ($P > 0.05$).

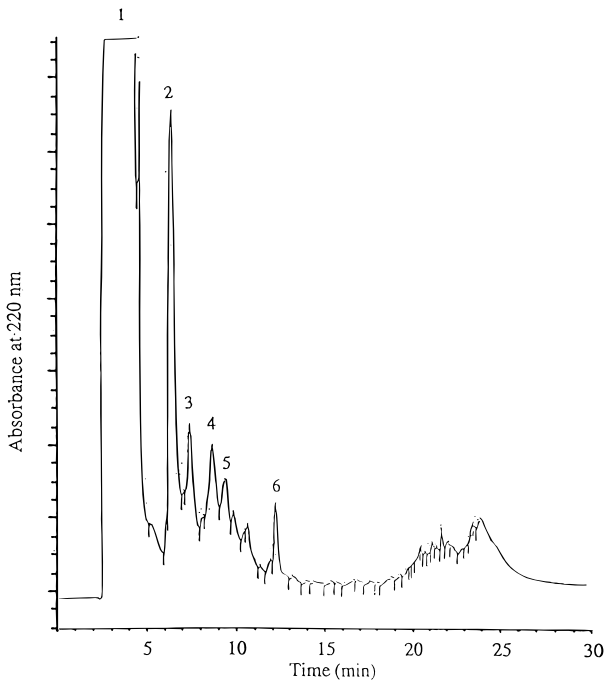

Figure 2. Analytical HPLC separation profile of the first eluant fraction collected from preparative HPLC. The injected sample was a molecular weight fraction between 500 and 3000 molecular weight from cheese 1. The numbers identify peaks that were collected for further analysis.

Analytical HPLC Analysis and Sensory Tasting. Because a variety of compounds could be contained in an eluant fraction collected over 5 min from preparative HPLC, peptides in eluant fractions 1, 2, 4, 9, and 12 (those with relatively high bitterness scores from Table 4) were further separated using analytical HPLC. Figure 2 contains an analytical HPLC separation chromatogram of the first eluant fraction collected from preparative HPLC where the initial sample was the molecular weight fraction between 500 and 3000 molecular weight from cheese 1. The fractions of the six peaks marked in Figure 2 were collected repeatedly, concentrated, reconstituted with water, and tasted by the sensory panel. The mean bitterness scores (in mm) of peaks 1–6 were 57.40, 7.43, 33.91, 44.80, 20.83, and 14.37, respectively. Results of the sensory data revealed no relationship between the bitterness intensity and

Table 5. Bitterness Scores and Amino Acid Molar Concentrations of 10 Bitter Peptide Fractions with Their Quantities Relative to Valine (Equal 1)

	bitter fraction (bitterness score[a])									
	1 (47.50)	2 (58.63)	3 (51.00)	4 (57.40)	5 (44.80)	6 (62.10)	7 (43.60)	8 (50.38)	9 (42.63)	10 (40.90)
glutamic acid/glutamine	14.59	6.77	33.17	26.50	19.58	24.82	7.42	1.22	11.63	10.67
aspartic acid/asparagine	0.92	1.43	1.88	1.75	4.12	3.18	1.92	0.16	0.63	0.50
glycine	0.41	0.20	1.38	3.75	3.79	0.35	*b*	0.01	*b*	1.29
serine	11.04	1.33	4.50	3.25	10.03	5.35	2.67	0.15	6.00	2.75
threonine	0.51	1.23	1.00	1.00	3.27	0.94	1.83	*b*	0.13	0.04
histidine	0.44	0.83	0.13	0.50	0.52	0.18	1.08	0.05	0.05	0.17
arginine	*b*	0.67	*b*	*b*	*b*	*b*	0.17	*b*	*b*	*b*
alanine	0.37	0.23	1.25	2.50	1.03	0.82	*b*	*b*	*b*	1.13
cysteine	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*
methionine	0.15	0.03	0.25	*b*	0.27	0.29	*b*	*b*	*b*	*b*
lysine	0.44	3.23	8.13	*b*	1.45	2.24	3.67	0.16	1.25	0.79
valine	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
leucine	1.74	0.70	0.50	0.75	3.36	3.06	1.00	*b*	2.50	1.58
proline	*b*	*b*	*b*	9.75	1.88	6.24	*b*	1.45	*b*	5.58
phenylalanine	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*
tyrosine	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*
isoleucine	0.37	0.87	7.75	0.75	3.36	0.53	1.33	*b*	*b*	1.67

[a] Bitterness score was measured in mm. [b] Data are not available because of nonmeasurable levels.

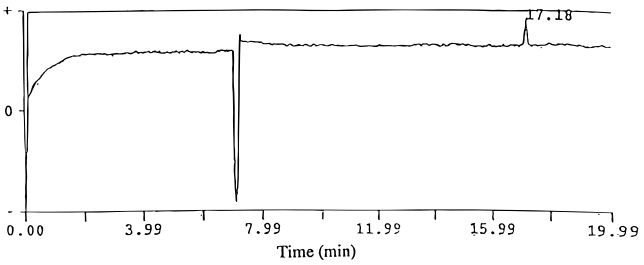

Figure 3. Capillary electrophoresis electropherogram of collected peak 4 from Figure 2.

peak elution order. Eluant of peak 1 was selected for subsequent analysis because it was significantly more bitter ($P < 0.05$) compared to other peaks. Peak eluants 3 and 4 also were collected for analysis because of their relatively high bitterness intensity.

Purity Confirmation of Isolated Peak Eluant. Purity of the isolated bitter peak eluants from analytical HPLC analysis was examined using capillary electrophoresis to ensure each peak contained only a single peptide. A capillary electrophoresis electropherogram of a purified HPLC peak fraction (peak 4 in Figure 2) is shown in Figure 3. The negative peak resulted from a potential change during the run. The single peak at about 17 min and the absence of other peaks indicated the purity of the injected sample. If there is more than one peak in the electropherogram, the injected sample needs to be repurified under different HPLC elution conditions before submitting into the amino acid analyzer. Because separation principles between open-tube capillary electrophoresis and HPLC are profoundly different, correlations between the peak elution time in capillary electrophoresis and the retention time in HPLC do not exist.

Amino Acid Composition of the Isolated Bitter Peptides. The bitterness scores and amino acid molar compositions of 10 isolated bitter peptide fractions with their quantities relative to valine (equal to 1) are given in Table 5. The order of amino acids is listed from the most hydrophilic to the most hydrophobic residue (from glutamic acid/glutamine to isoleucine). Tryptophan is not shown in the table because it was decomposed by the hydrolysis with HCl. The relative hydrophilic amino acid residues such as glutamic acid/glutamine, aspartic acid/asparagine, glycine, serine, threonine, histidine, and lysine were detected in almost every isolated bitter peptide, although some residual amino acid residues might result from contaminations during the collection from HPLC. Among the hydrophilic amino acids, glutamic acid or glutamine was found at high levels compared to the other amino acids. Serine, with the second highest quantity in general, might be another important hydrophilic residue in the bitter peptide composition. Lysine, a basic amino acid, had the second highest quantity in peptides 2, 3, and 7. Except in peptide fractions 2 and 7, arginine was very limiting in the peptides. Cysteine was not detected in any of the isolated bitter peptides.

Relatively hydrophobic amino acids, valine, leucine, and isoleucine, were observed in most peptide compositions. Leucine was found at relatively high levels in peptides 1, 5, 6, and 9 and isoleucine in peptides 3 and 5. Proline was not prevalent in every bitter peptide fraction; however, it had a fairly high content in peptides 4, 6, and 10 compared to the other hydrophobic amino acid residues. In general, the amount of methionine was low, and both tyrosine and phenylalanine were absent in all the bitter peptide fractions.

Champion and Stanley (1982) reported that two hydrophobic amino acids, valine and leucine, occurred at higher levels in the HPLC-separated bitter fractions than in the nonbitter fractions. They also noted that amounts of glutamic acid, leucine, proline, and valine were high in two other HPLC fractions which resembled the bitter fraction from gel filtration. However, the findings only reflected an overall sensory effect of bitterness from eluants which contained a mixture of peptides and amino acids. Bitter peptides that had relatively large amounts of glutamic acid, proline, leucine, and valine but small amounts of histidine, tyrosine, and methionine were isolated from Cheddar cheese by Edwards and Kosikowski (1983). These results are in close agreement with the present findings in Table 5. Water extraction was used in their study to fractionate water-soluble nitrogen that contained bitter-tasting peptides from cheese. This might explain why some bitter peptides had relatively high levels of acidic and hydroxyl amino acids, normally more hydrophilic, and low levels of aromatic amino acids, more hydrophobic. Using water extraction followed by series of separation steps, three bitter peptides were isolated from Cheddar cheese by Richardson and Creamer (1973). With the sequence identified and average hydro-

phobicity calculated, two out of three bitter peptides were found to be fairly hydrophilic because of the high content of acidic amino acids. Although some similarities were noted between the results in Table 5 and the findings of previous researchers, information regarding the relative hydrophobicity of the isolated bitter peptides was not obtained in our study because amino acid analysis only revealed the residue composition of peptides, not the sequence.

New information presented by our study is that there are likely a large number of bitter peptides responsible for bitter cheese and the degree of hydrophobic similarity among these peptides may not be as great as previously thought.

Conclusions. The analysis of Cheddar cheese samples showed that the composition of cheese may have some influence on bitterness intensity, but it is not a determinant factor. Bitterness was found in all fractions of cheese extracts that were fractionated by molecular weight, but the bitterness intensity was not significantly different in the fractions with a molecular weight between 500 and 3000 and >3000. When a bitter-tasting fraction was incorporated into cheese, the increased bitterness intensity was more significant in reduced-fat cheese than in full-fat cheese. This could be attributed to the effect of fat in bitterness masking. There are no trends between the bitterness intensity and the order of elution in HPLC analyses, suggesting that the chemical characteristics of bitter peptides of interest could be quite different from each other in polarity and molecular weight. Composition analysis of isolated bitter peptides showed relatively high contents of hydrophilic amino acid residues, especially acidic and hydroxyl amino acids. Several hydrophobic amino acids were high in some peptide fractions, indicating that both hydrophilic and hydrophobic peptides could be bitter.

LITERATURE CITED

Adler-Nissen, J. Control of the proteolytic reaction and of the level of bitterness in proteins hydrolysis processes. *J. Chem. Technol. Biotechnol.* **1984**, *34B*, 215−222.

Anliker, J. A.; Bartoshuk, L.; Ferris, A. M.; Hooks, L. D. Children's food preferences and genetic sensitivity to the bitter taste of 6-*n*-propylthiouracil (PROP). *Am. J. Clin. Nutr.* **1991**, *54*, 316−320.

AOAC. *Official Methods of Analysis*, 14th ed.; Association of Official Analytical Chemists International: Arlington, VA, 1984.

Aston, J. W.; Creamer, L. K. Contribution of the components of the water soluble fraction to the flavour of Cheddar cheese. *N. Z. J. Dairy Sci. Technol.* **1986**, *21*, 229−248.

Champion, H. M.; Stanley, D. W. HPLC separation of bitter peptides from Cheddar cheese. *Can. Inst. Food Sci. Technol. J.* **1982**, *15*, 283−288.

Clegg, K. M.; Lim, C. L.; Manson, W. The structure of a bitter peptide derived from casein by digestion with papain. *J. Dairy Res.* **1974**, *41*, 283−287.

Deane, D. D.; Dolan, E. T. Curing of cheese with reduced fat content. *J. Dairy Sci.* **1973**, *56*, 631−633.

Edwards, J.; Kosikowski, F. V. Bitter compounds from Cheddar cheese. *J. Dairy Sci.* **1983**, *66*, 727−734.

Glanville, E. V.; Kaplan, A. R. Food preference and sensitivity of taste for bitter compounds. *Nature* **1965**, *2*, 851−853.

Grossman, P. D.; Colbum, J. C.; Lauer, H. H. Application of free-solution capillary electrophoresis to the analytical scale separation of proteins and peptides. *Anal. Chem.* **1989**, *61*, 1186−1194.

Hamilton, J. S.; Hill, R. D.; Leeuwen, H. V. A bitter peptide from Cheddar cheese. *Agric. Biol. Chem.* **1974**, *38* (2), 375−379.

Harwalkar, V. R.; Elliott, J. A. Isolation of bitter and astringent fractions from Cheddar cheese. *J. Dairy Sci.* **1971**, *54*, 8−11.

Kuchroo, C. N.; Fox, P. F. Soluble nitrogen in Cheddar cheese: Comparison of extraction procedures. *Milchwissenschaft* **1982**, *37*, 331−335.

Lee, K. D.; Warthesen, J. J. Mobile phases in reverse-phase HPLC for the determination of bitter peptides in cheese. *J. Food Sci.* **1996**, in press.

Lowrie, R. J.; Lawrence, R. C. Cheddar cheese flavour. IV. A new hypothesis to account for the development of bitterness. *N. Z. J. Dairy Sci. Technol.* **1972**, *7*, 51−53.

McCormick, R. M. Capillary zone electrophoretic separation of peptides and proteins using low pH buffers in modified silica capillaries. *Anal. Chem.* **1988**, *60*, 2322−2327.

Olson, N. Making a marketable low-fat cheese. *Dairy Rec.* **1984**, *85* (2), 115.

Richardson, B. C.; Creamer, L. K. Casein proteolysis and bitter peptides in Cheddar cheese. *N. Z. J. Dairy Sci. Technol.* **1973**, *8*, 46−51.

SAS User Guide: Statistics, version 5; SAS Institute Inc.: Cary, NC, 1985.

Singh, T. K.; Fox, P. F.; Hojrup, P.; Healy, A. A scheme for the fractionation of cheese nitrogen and identification of principal peptides. *Int. Dairy J.* **1994**, *4*, 111−122.

Sullivan, J. J.; Jago, G. R. The structure of bitter peptides and their formation from casein. *Aust. J. Dairy Technol.* **1972**, *27*, 98−104.

Thomas, T. D.; Pearce, K. N. Influence of salt on lactose fermentation and proteolysis in Cheddar cheese. *N. Z. J. Dairy Sci. Technol.* **1981**, *16*, 253−259.

Visser, S.; Hup, G.; Exterkate, F. A.; Stadhouders, J. Bitter flavour in cheese. 2. Model studies on the formation and degradation of bitter peptides by proteolytic enzymes from calf rennet, starter cells and starter cell fractions. *Neth. Milk Dairy J.* **1983**, *37*, 169−180.

Received for review May 23, 1995. Revised manuscript received January 23, 1996. Accepted February 20, 1996.[⊗] Support was provided by the Minnesota−South Dakota Dairy Foods Research Center.

JF950521J

[⊗] Abstract published in *Advance ACS Abstracts*, April 1, 1996.